Optimization of Culture Conditions for Production of β-Mannanase by Strain *Nonomuraea* sp. ID06-379 using Submerged Substrate Fermentation

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Abstract

The objective of this study was to investigate the effect of media compositions on the production of β-mannanase by *Nonomuraea* sp. ID06-379. The study was focused on the influence of carbon, nitrogen, phosphorus and detergents on β-mannanase synthesis through manipulating media compositions on production medium. The results indicated that for carbon sources, locus bean gum (0.745 ± 0.036 U/ml) showed maximum mannanase activity. Malt extract was the best nitrogen source for producing β-mannanase (1.075 ± 0.006 U/ml), $(NH_4)_2HPO_4$ as phosphate source (1.733 ± 0.026 U/ml) and Tween 80 (1.145 ± 0.003 U/ml) as surfactants effect on increasing permeability of bacterial cell membrane, enhancing membrane transport and excretion of extracellular enzymes into the production media. The results showed that 1% malt extract, 0.5% locus bean gum and 0.05% $(NH_4)_2HPO_4$ were good substances for nitrogen source, carbon source and phosphate respectively. The highest production of β-mannanase by *Nonomuraea* sp. ID06-379 (5.33 U/mg) was reached in the medium optimization (Vogel's minimal medium) contained the following ingredients: 0.5% locus bean gum, 1% malt extract and 0.05% $(NH_4)_2HPO_4$, under submerged fermentation with shaking at 120 rpm and 28°C for 2 days incubation.

Keywords: β-mannanase, actinomycetes, *Nonomuraea* sp. ID06-379, optimization medium, submerged substrate fermentation.

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Introduction

Hemicelluloses are polysaccharides consisting of linear and branched chain in the cell walls of higher plants which are closely associated to the cellulose and lignin forming lignocelluloses biomass (Moreira & Filho, 2008). Mannans are hemicelluloses, accounting for 15 to 20% dry (basis) in softwoods and only 5% in hardwoods. The main chain of softwood mannan is composed of β-1,4-linked-D-mannopyranose and D-glucopyranose units. The residues in the main chain are partially substituted by α-1,6-linked-D-galactosyl side groups. Mannans can be obtained from legume seeds, coconut kernel, palm kernel, coffee beans, tubers of konjac, and copra (Lin & Chen 2004). Indonesia is an industrial based country and every year tonnes of agricultural wastes such as described above are accumulated, and these mannan rich materials are potentially to be used as substrate in fermentation for the production of enzymes and other microbial secondary metabolites.

Mannanases are used mainly for improving the quality of food, feed and aiding in enzymatic bleaching of soft- wood pulps in the paper and pulp industries. Products of mannan hydrolysis (mannose and oligossacharides) may be used both in the food and feed industry, as prebiotic additives and in the pharmaceutical industry. As it hydrolyzes, mannan may also be converted into liquid fuel. Complete mannan hydrolysis requires the action of many

enzymes, such as endo-1,4-β-mannanases (β-mannanases), which are crucial for depolymerization (Dhawan & Kaur, 2007).

The growing interest in mannanases production for industrial applications is due to its importance in the bioconversion of agro-industrial residues. Certain fungi and bacteria, mostly soil-living, produce hydrolytic enzymes that are able to degrade lignocellulosic substrates. During the last decade, researchers have been largely interested in the potential biotechnological uses of mannanases.

Production of mannanases by microorganisms is more promising due to its low cost, high yield and readily controlled conditions (Meenakshi *et al.,* 2010). Fermentation is the primary technique for the production of various enzymes. Submerged substrate fermentation is usually implemented in case of bacterial enzyme production, due to the requirement of higher water potential (Rashid *et al*., 2012).

Mannanases of microbial origin have been reported to be both induced as well as constitutive enzymes and are usually been secreted extracellular into the medium in which the microorganism is cultured. Submerged substrate fermentation utilizes free flowing liquid substrates, and the bioactive compounds are secreted into the fermentation broth. This fermentation technique is best suited for microorganisms such as bacteria that require high moisture. Submerged substrate fermentation owing to better monitoring and ease of handling and an additional advantage of this technique is that purification of products is easier. Submerged substrate fermentation is primarily used in the extraction of secondary metabolites that need to be used in liquid form. A range of strategies can be used to manipulate the physical, environmental, and nutritional conditions under submerged fermentation for increased production efficiency as well as optimal mannanase production (Jain *et al*., 2013).

Mannanases (EC 3.2.2.78 endo-1,4-β-D-mannan mannanohydrolases) occur widely in microorganisms in bacteria, yeasts and fungi as well as from germinating seeds of terrestrial plants (Ferreira & Filho, 2004; Heck *et al*., 2005; Jiang *et al*., 2006; Lin *et al*., 2007). Actinomycetes are known for their ability to degrade recalcitrant polymers such as lignocellulose. Species such as *Streptomyces, Nocardia, Arthrobacter* and *Rhodococus*

produce multiple hemicellulases, cellulases and lignases that play a pivotal role in lignocellulose degradation (Khanderparker & Numan, 2008). *Nonomuraea* sp. ID06-379 is gram positive, soil-inhabiting actinomycetes with broad degradative activity on plant cell wall constituents. *Nonomuraea* sp. ID06-379 can utilizes locus bean gum as the major carbon sources and secretes mannanases enzymes. To date no published data about hemicellulolytic system of *Nonomuraea.*

During a previous study we screened a number of mannanolytic actinomycetes from leaf and litter samples in several areas in Indonesia. One of the strains, *Nonomuraea* sp. ID06-379, was found to show a good mannanolytic activity (unpublished data).

The objective of this research was to study and optimize culture condition for production of mannanase by *Nonomuraea* sp. ID06-379 strain under submerged substrate fermentation.

Materials and Methods

Microorganism and maintenance. An actinomycete *Nonomuraea* sp. ID06-379 capable of producing extracellular β-mannanase was isolated from soil of Kutai soil (Widyastuti & Ando 2009) and a collection from Biotechnology Culture Collection (BTCC), Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI). *Nonomuraea* sp. ID06-379 was maintained on ISP-2 agar medium with the following nutrient 0.4% Yeast Extract, 0.1% malt extract and 4% glucose. Nystatin (50 µg/ml) was added as antifungal.

Media preparation and enzyme production. For the production of β-mannanase in submerged state fermentation, the isolate was grown at 28°C in 250 ml Erlenmeyer flask in Vogel's minimal medium modified by Chellapandi and Jani (2008). This medium contained the following ingredients (g/l): Water, 750 ml; Na_3 citrate.$2H_2O$, 130 g; KNO_3, 126 g; $(NH_4)H_2PO4$, 144 g; KH_2PO_4, 80 g; $MgSO_4.7H_2O$, 10 g; $CaCl_2.2H_2O$, 5g in 20 ml distilled water, added drop wise; trace elements solution, 5 ml; biotin solution (0.1 mg/ml), 2.5 ml and no adjustment of pH was necessary or desirable. Trace elements have composition of following recipe: Distilled water, 95 ml; Citric acid.H_2O, 5 g; $ZnSO_4.7H_2O$ 5 g; $Fe(NH_4)_2(SO_4)_2.6 H_2O$, 1 g; $CuSO_4.5 H_2O$, 250

mg; MnSO$_4$.H$_2$O, 50 mg; H$_3$BO$_3$, 50 mg; Na$_2$MoO$_4$.2H$_2$O, 50 mg. This medium was prepared separately, autoclaved and stored at 4°C in a refrigerator until the use. All flasks were autoclaved at 121°C for 20 min then cooled to room temperature. After cooling, media were inoculated with 10% of bacterial inoculum pre-grown for 3 days. Flasks containing inoculated media were incubated at room temperature on a rotary shaker at 120 rpm for 5 days incubation. The culture broth was withdrawn aseptically at periodic intervals, and then centrifuged at 8,000 rpm for 15 min. The culture supernatant was used as enzyme source.

Determination of β-mannanase producing actinomycete on several substrate specific mannan and agro source containing mannan. Isolate of *Nonomuraea* sp. ID06-379 was cultured on ISP-2 medium containing 0.5% LBG (pH 7.0) and incubated for 4 days at 28°C with shaking (120 rpm). The culture broth was harvested by centrifugation at 8,000 rpm, 4oC for 15 min. The culture supernatant was then concentrated 30 fold using Vivaspin 20 (Sartorius) 5kDa molecular-weight cutoff. In this determination method, we are using two layers agar plate. Bottom layer is containing 50 mM acetic acid buffer (sodium acetate + acetic acid) pH 6.5 with 1.7% agar. Upper layer is containing 0.5% substrate (azo carob β-mannan (Megazyme), locus bean gum (Sigma), konjac glucomannan (Megazyme), elephant foot yam (*Amorphophallus oncophyllus*) fluor, porang (*A. muelleri*) fluor, pollard, soybean meal, palm kernel cake, or copra meal), 1.7% agar in 50 mM acetic acid buffer pH 6.5. As much as 3 µl, 5 µl, 7 µl, and 10 µl of culture supernatant (concentrated 30 folds) then drops onto each substrate agar plate. Dried up the plate for 15 min at room temperature, and then incubated at 37oC for 3 days. Plates were then flooded with 0.1% Congo red for 15-20 min, then washed with 1 mM NaCl. β-Mannanase production was indicated by the appearance of a pale halo with orange edges, indicative of areas of hydrolysis (Carder, 1986). This halo was measured for subsequent calculation of the enzymatic index (EI) using the equation:

$$EI = \frac{\text{diameter of hydrolysis zone}}{\text{diameter of colony}}$$

The strains that showed an EI higher than 1.50 were considered to be potential producers of mannanases (Florencio *et al.*, 2012).

Effect of different carbon, nitrogen, phosphate sources and surfactant on β-mannanase activity. A study on the influence of different nutrients include carbon, nitrogen, phosphorus and surfactants on mannanase synthesis was examined by replacing appropriate sources in the 250 ml flask containing 50 ml of the fermentation medium. The fermentation medium was prepared by adding 20 ml Vogel's minimal medium, 3 g yeast extract and 10 g locus bean gum, and finally made up to 1 L with distilled water. The appropriate carbon source for β-mannanase production by the *Nonomuraea* sp. ID06-379 was determined by replacing LBG as carbon source in the fermentation medium and supplementing with different carbon source (mannose, glucoce, maltose, lactose, xylose, fructose, sucrose, inulin, LBG, porang fluor and palm kernel cake) at 1% (w/v) level carbon and as a control was the same fermentation medium without carbon source. Different nitrogen sources (malt extract, peptone from casein, tryptone, yeast extract, casein, meat extract, (NH$_4$)$_2$HPO$_4$, (NH$_4$)$_2$SO$_4$, KNO$_3$) at 0.3% level for organic nitrogen and 0.03% for inorganic nitrogen, replacing the prescribed yeast extract of the fermentation medium. Different phosphate sources (0.03%, w/v of (NH$_4$)$_2$HPO$_4$, Na$_2$HPO$_4$, KH$_2$PO$_4$ or K$_2$HPO$_4$) was supplemented in the fermentation medium. Effect of different surfactants (0.003%, w/v Tween 80 or SDS) in fermentation medium was also investigated for mannanase production by *Nonomuraea* sp. ID06-379. Mannanase production was studied by incubating culture in fermentation medium under shaking condition at 30°C for 3 days incubation.

Effect of different concentration of locus bean gum, malt extract and (NH$_4$)$_2$HPO$_4$ on β-mannanase production. Carbon (locus bean gum), nitrogen source (malt extract) and inducer (NH$_4$)$_2$HPO$_4$ were also evaluated for optimal concentrations in the range of 0 to 2% (w/v) using fermentation medium. Mannanase production was studied by incubating culture in fermentation medium under shaking condition at 30°C for 3 days incubation. The modified optimization medium consisting of control medium supplemented with 0.5% (w/v) LBG,

1.0% (w/v) malt extract, and 0.05% (w/v) $(NH_4)_2HPO_4$, was evaluated for β-mannanase production compared to control medium (Vogel's minimal medium). Total soluble protein in the culture filtrate was determined using the Bio-Rad Protein Assay Kit based on the method of Bradford (1976). The concentration was determined based on the reference protein standard curve using bovine serum. The biomass was estimated by determining the dry weight of the biomass after it had been dried to constant weight in an oven at 80°C.

Determination of β-mannanase activity.
Mannanase, activity was assayed by measuring the release of reducing sugar from locust bean gum (LBG), following the dinitrosalicylic acid (DNS) method (Miller, 1959). β-Mannanase activity was determined by using 0.5% (w/v) LBG as substrate. The enzyme sample (0.1 ml) was incubated with 0.9 ml of the substrate solution at 50ºC for 20 min. The amount of reducing sugars produced in the enzyme reaction was measured as D-mannose reducing equivalents by the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). Substrate and enzyme controls were used with addition of distilled water instead of the enzyme or substrate, respectively. Reducing sugar concentration was determined using the DNS method. Results were interpreted in terms of enzyme activity in which one unit (U) of enzyme activity was defined as the amount of enzyme which liberates 1 μmol of mannose per minute under the given assay conditions (Miller, 1959). Standard mannose (reducing sugar) at a concentration in the range of 0.25-8.00 mg/g was prepared. The concentration of the reducing sugar was determined based on a standard curve. Protein assay was determined using the Bio-Rad Protein Assay Kit based on the method of Bradford (1976). The concentration was determined based on the reference protein standard curve using bovine serum.

Results and Discussion

Based on mannanolytic activity in cell-free culture supernatant as assayed by double layer, *Nonomuraea* sp. ID06-379 isolate was then assayed on different commercial mannan substrates and agroindustry mannan sources.

The spots of culture supernatant of *Nonomuraea* sp. ID06-379 that concentrated 30 fold using Vivaspin 20 (Sartorius) 5kDa molecular-weight cutoff for higher and concentrated enzyme activity onto double layer containing substrate mannan plates were shown in Figure 1.

The effect of different carbon sources of commercial mannans (Azo carob mannan, LBG and konjac glucomannan), and several agroindustry substances containing mannan include elephant foot yam flour, porang flour, pollard, soybean meal, palm kernel cake and copra meal, on the production of β-mannanase from *Nonomuraea* sp. ID06-379 was evaluated. Mannanase produced by *Nonomuraea* sp. ID06-379 fermentation showed a good hydrolysis activity on different samples of mannan. Of all the mannan sources investigated, the mannanase *Nonomuraea* sp. ID06-379 produce diffusion of enzyme into the substrate, hence produces hydrolysed zone which can be visualised by staining non-degraded substrate with Congo red dye (Carder, 1986) showed clear zones against red colour of non-hydrolysed medium (Figure 1).

Mannanase produced by *Nonomuraea* sp. ID06-370 would be a real endo β- mannanase since this enzyme hydrolyze several type of mannans such as β-mannans (azo carob mannan), galactomannan (LBG, palm kernel cake,and copra meals), and glucomannans (konjac glucomannan, porang flour and elephant foot yam flour).

The method of the radial diffusion in solid media indicated that the activity in qualitative form, directly correlating the diameters of the halo of degradation and the colony. The Enzymatic Index (EI) was expressed by the relationship between the average diameter of the degradation halo and the average diameter of the colony growth (Carrim *et al.*, 2006). The Enzymatic Index is a practical tool that facilitates and speeds the selection and the comparison of the enzymatic degradation on different substrates from the isolate, once in this index direct consideration the correlation are taken between the size of the halo and the degradation capacity of the microorganisms and suggested that Enzymatic Index larger than 1.0 were indicative of excretion of enzymes (Lin *et al.*, 1991). From Table 1, it was observed that all EI value of β-mannanase from *Nonomuraea* sp. ID06-379 was larger than 1.0 at all substrates tested, indicative that

Nonomuraea sp. ID06-379 had been suggested as a producer of extracellular mannanase. In the results obtained it was observed that β-mannanase from *Nonomuraea* sp. ID06-379 demonstrated the best EI for substrate Azo carob manan (2.7) from commercial substrate, this medium was specific for detection of endo β-mannanase and from agrowaste substrate soybean meal (3.9) showed the highest EI value.

Table 1 also shows that β-mannanase from *Nonomuraea* sp. ID06-379 was able to degrade substrates mannan in various types: linear mannan (Azo carob mannan, pollard, soy bean meal), galactomannan (LBG, palm kernel cake and copra meal) and glucomannan (konjac glucomannan, elephant foot yam fluor and porang fluor).
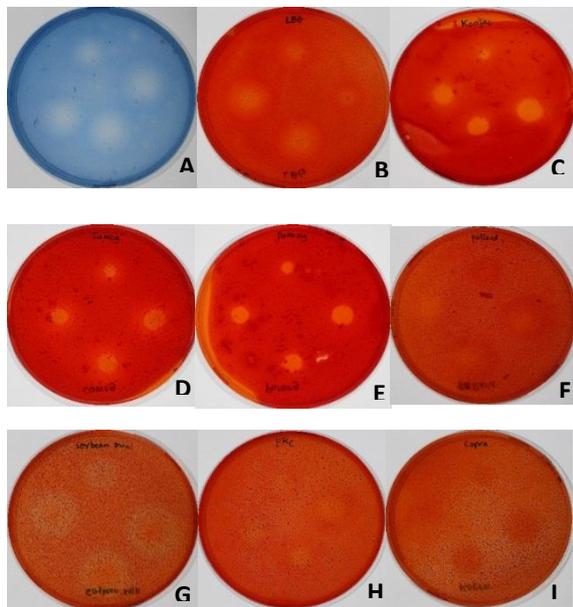

Figure 1. Degradation of several mannan substrate by β-mannanase *Nonomuraea* ID06-379. (A) azo carob mannan, (B) locus bean gum, (C) konjac glucomannan, (D) elephant foot yam (suweg, Indonesian) flour, (E) porang (*Amarphopallus oncophilus*) flour, (F) pollard, (G) soybean meal, (H) palm kernel cake, (I) copra meal. Counter clock wise of the spot from top was volume of enzyme spotted: 3 μl, 5 μl, 7 μ, and 10 μl.

To optimize mannanase yield, varying carbon sources were augmented into production medium, and the results are presented in Figure 2. Almost all the carbon sources tested support the growth of *Nonomuraea* sp. ID06-379, except xylose and inulin. Among commercial carbon sources, LBG (1.0% w/v) was found to induce maximum production of β-mannanase (0.745 U/mg), followed by glucose (0.728 U/ml) and porang flour (0.709 U/mg). The palm kernel cake (PKC) favored the growth but less mannanase was produced. The simple sugars supported the growth but did not induce the mannanase production. Higher β-mannanase was obtained when LBG is used as a sole carbon source. Similar result was obtained by *Bacillus* sp. mg 33 (Meenakshi *et al.* 2010), *Bacillus circulans* CGMCC1554 (Yang *et al.* 2009), and alkalophilic *Bacillus* species (Zhao *et al*., 2011).

Table 1. Enzymatic activity of *Nonomuraea* sp. ID06-379 on several mannan substrates

Substrate mannan	Enzymatic Index (EI)[*]
Azo carob mannan	2.7
Locus bean gum	2.2
Konjac glucomannan	1.7
Elephant foot yam fluor	1.4
Porang fluor	1.4
Pollard	3
Soybean meal	3.9
Palm kernel cake	1.9
Copra meal	2

[*]The enzymatic index represents the halo diameter of degradation/volume of enzyme in μl.

Simple sugar such as sucrose, lactose, and mannose as carbon sources did not show significant improvement on enzyme productivity by *Nonomuraea* sp. ID06-379. Xylose and inulin were repressed of β-mannanase production by this isolate. Glucose and fructose exhibited a good inducer to enzyme yield.

The synthesis of β-mannanase is necessary for the degradation of energy-yielding polymeric materials as lignocelluloses. It is inducible in microbial cells and appears to be controlled by carbon repression when more easily metabolizable carbon sources e.g. glucose is present in the culture medium together with a substrate suitable for inducing mannanase (Chantorn *et al*., 2013). The extracellular β-mannanase synthesis is cyclic dependent and therefore subject to catabolite repression by soluble sugar accumulation. Only some soluble sugars as reported here are suggested responsible for increasing enzyme synthesis in the production media (Lynd *et al*., 2002).

Pokhrel & Ohga (2007) found that carbon source was independently responsible in

mycelial growth and polysaccharide production. So that, even if same species needs a different carbon source for specific metabolite production, it is possible that different carbon source might have different effect on catabolic repression on the cellular secondary metabolism (Aziz, 2008).
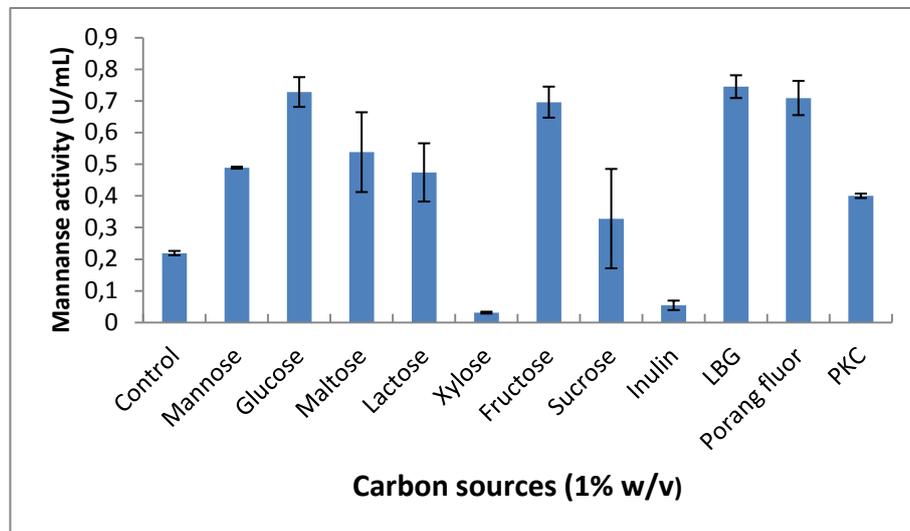

Figure 2. Effect of varying carbon sources (1%, w/v) on mannanase production by *Nonomuraea* sp. ID06-379 isolate, grown under shaking condition at 3 days incubation

The different organic and inorganic nitrogen sources were evaluated for β-mannanase production by *Nonomuraea* sp. ID06-379. The fermentation medium was supplemented with each of these nitrogen sources at a level of 0.3% for organic nitrogen sources and 0.03% for inorganic nitrogen sources.

Mannanase activity in the culture supernatant was differently affected by the nature of nitrogen source (Figure 3). The all tested nitrogen sources were able to support the production of β-mannanase but did not show so much improvement on enzyme productivity. Regarding the effect of different nitrogen sources on the mannanase activity by *Nonomuraea* sp. ID06-379 it was found that the use of malt extract (1.075 U/ml)) was best followed by yeast extract, while other organic nitrogen sources yielded β-mannanase with all liberating around 0.243-0.399 U/ml. The lowest β-mannanase production was obtained in ammonium sulphate $(NH_4)_2SO_4$. β-Mannanase production was found to be maximum when *Nonomuraea* sp. ID06-379 was grown on medium containing malt extract. The Vogel's medium supplemented with malt extract as nitrogen source in the fermentation medium yielded maximum β-mannanase activity (1.075 U/ml).

We found that malt extract served as intensive sources to *Nonomuraea* sp. ID06-379 β-mannanase production. Yeast extract also contributed for enhancing production of this enzyme by *Nonomuraea* sp. ID06-379. Urea and yeast extract was the best nitrogen source for mannanase production and growth by *B. subtilis* IE and *B. megaterium* UI (Adebayo-Tayo *et al.*, 2013), whereas ammonium chloride for filamentous fungi are reported as potential nitrogen sources for mannanase production of *Aspergillus niger* (Youseff *et al.*, 2006). Differences in enzyme production by the use of different nitrogen sources may be due to different requirement by the isolates. Similar results have been reported from *Bacillus* sp. N16-5 with yeast extract (Lin *et al.*, 2007), *Bacillus* sp. by polypeptone (Zhang *et al.*, 2011) as suitable as nitrogen sources. Among nitrogen sources, organic nitrogen enhanced mannanase production more than the inorganic sources. This is similar to the results of Kote *et al.* (2009).
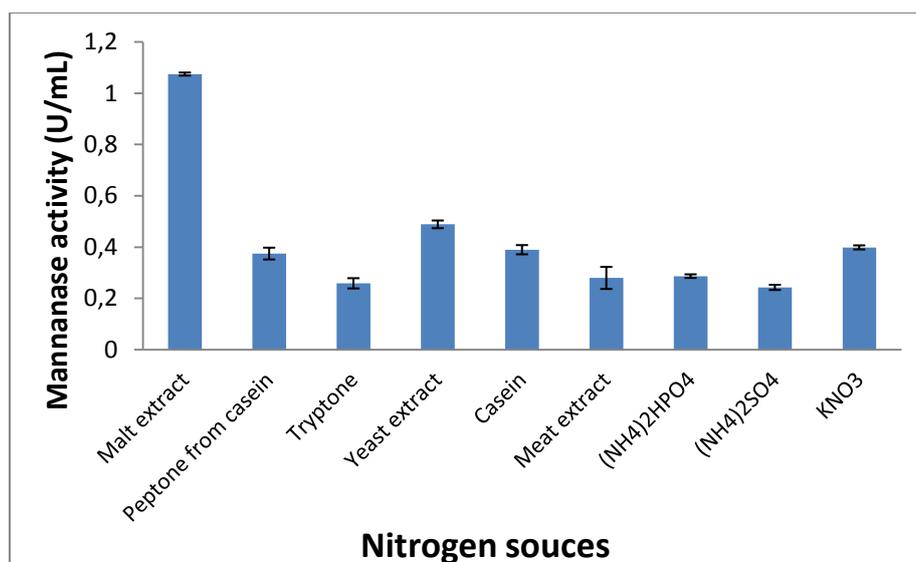
Figure 3. Effect of different nitrogen sources on β-mannanase production by *Nonomuraea* sp. ID06-379 isolate under shaking condition at 3 days incubation. The concentration of organic nitrogen and inorganic nitrogen sources was 0.3% and 0.03% (w/v), respectively

The effect of different inorganic phosphate sources on producing β-mannanase was examined (Table 2) $(NH_4)_2HPO_4$ for *Nonomuraea* sp. ID06-379 found to be good for improved β-mannanase activity.

Enzyme synthesis by the isolate improved in culture broth when incorporated with K_2HPO_4, KH_2PO_4 and Na_2HPO_4. It suggested that adding relevant concentrations nutrient supplements are desirable to bring a favorable condition for *Nonomuraea* isolate to increase β-mannanase yield.

Table 2. Effect of different phosphate sources (0.03%, w/v) on β-mannanase production by *Nonomuraea* sp. ID06-379 isolate under shaking condition at 3 days incubation.

Phosphate source	Mannanase activity (U/ml)
$(NH_4)_2HPO_4$	1.733 ± 0.026
Na_2HPO_4	1.408 ± 0.034
KH_2PO_4	1.524 ± 0.018
K_2HPO_4	1.604 ± 0.144

Surfactants are considered as good inductive sources while production media optimization. Surfactants are used to increase the permeability of bacterial cell membrane by which enhancing membrane transport and excretion of extracellular enzymes into the production media (Chantorn *et al*., 2013). Consequently, the maximum β-mannanase production was achieved (1.145 U/ml) at 3 days when the production medium supplemented

with Tween-80 (Table 3). SDS not exhibited a stimulatory effect for mannanase production.

Table 3. Effect of different surfactants (0.003%, w/v) on mannanase production by *Nonomuraea* sp. ID06-379 isolate under shaking condition at 3 days incubation.

Surfactant	Mannanase activity (U/ml)
Tween 80	1.145 ± 0.003
SDS	0.167 ± 0.005

Effect of different concentration of locus bean gum, malt extract and $(NH_4)_2HPO_4$ on β-mannanase production

Mannanase production of *Nonomuraea* sp. ID06-379 isolate on different concentration of carbon, nitrogen and phosphate source was measured according to their growth in 3 days incubation. Figure 4 showed that the isolate was able to produce extracellular β-mannanase when cultured in liquid medium containing locus bean gum as sole carbon source, malt extract as nitrogen source and $(NH_4)_2HPO_4$ as phosphate source. Each concentration of nutrients sources tested exhibit different pattern of β-mannanase production.

Locus bean gum; and malt extract; were selected for further evaluating the optimal concentrations in the range from 0 to 2% (w/v) and $(NH_4)_2HPO_4$ in the range 0.025 to 0.4% (w/v). The highest specific β-mannanase activity of 1.86 U/mg protein on 3 days of the cultivation with 0.5% locus bean gum supplemented medium was achieved (Figure

4A), whereas, 1% malt extract, and 0.05% $(NH_4)_2HPO_4$, which were the best sources of carbon, organic nitrogen, and phosphate, respectively, gave specific enzyme activities of 4.04, and 3.86 U/mg protein on 3 days of the cultivation as shown in Figures 4A-4C, respectively.
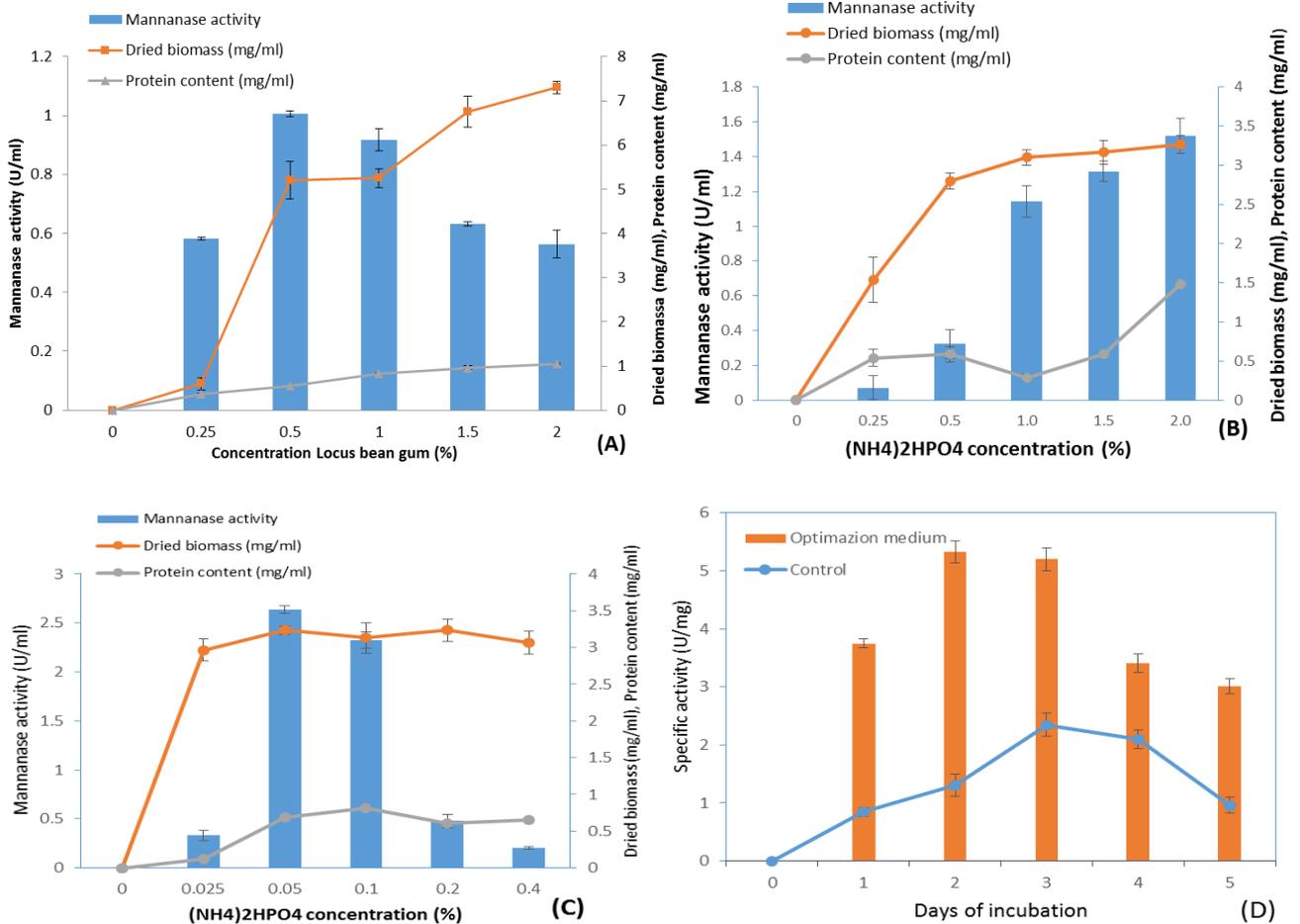

Figure 4. Production of β-mannanase by *Nonomuraea* sp. ID06-379 at different concentration of locus bean gum (A), malt extract (B) and ammonium phosphate (C). Specific activity of mannanase production with optimization medium comparing to that of control medium (D).

Figure 4 shows that *Nonomuraea* sp. ID06-379 had enhanced mannanase production in the optimization medium. The enzyme production reached a maximum activity after 2 days compared to 3 days control using Vogel's minimal medium (Figure 4D). The highest production of β-mannanase was 5.33 U/mg protein on day 2 of the cultivation time.

It was observed that enzyme production increased with increasing concentration LBG up to 0.5%. and subsequent increase substrate concentration resulted into gradual decrease in enzyme production. Low substrate concentration results in an increase in yield and reaction rate of the hydrolysis while, high substrate concentration can cause substrate inhibition, which substantially lowers enzyme formation (Liu & Yang, 2007; Singhania *et al.*, 2007). Locus bean gum at 0.5% level proved to be the best for β-mannanase production by *Nonomuraea* sp. ID06-379. This result was in conformity with other reports. The optimum LBG concentration for β-mannanase production by a strain of *Paenibacillus* sp. MSL-9 was 0.3% (Manjula *et al.*, 2010).

Figure 4B show the different concentrations of malt extract was added separately to Vogel's minimal medium and evaluated individually. Malt extract as nitrogen source concentrations of 0.25%, 0.5%, 1% and 2% were used and

maximum β-mannanase activity of 1.52 U/ml was obtained at 2% malt extract concentration. The optimum nitrogen source for β-mannanase production by a strain of fungi *Trichosporonoides oedocephalis* in submerged fermentation was 0.2% ammonium nitrate gave maximum enzyme activity and yield. The result obtained could be attributed to the fact that the ammonium nitrate provided both the ammonium as well as sulphate ions for conidial cell growth and enzyme production (Mekala *et al.*, 2008; Olaniyi *et al.*, 2013). Nitrogen source are usually used for protein synthesis substances more than growth.

Phosphate salts with defined concentration can promote the growth of organisms, and stimulate synthesis of a specific extracellular β-mannanase enzyme in production medium (Chellapandi & Jani, 2008). The maximum β-mannanase specific activity was achieved at concentration 0.05% of $(NH_4)_2HPO_4$ (3.86 U/mg). At higher or lower concentration, the enzyme activity showed a lower value. Similar results have been found in *Clostridium tertium* KT-5A, $(NH_4)_2HPO_4$ and $NH_4H_2PO_4$ at 0.2% concentration (w/v) were more effective for mannanase production in broth culture (Kataoka and Tokiwa, 1998).

Nonomuraea sp. ID06-379 with potential mannanolytic activity subjected to produce mannanase in liquid culture and a study on enzyme yield. Data presented in Figure 4D showed the effect of different incubation periods on β-mannanase production by *Nonomuraea* sp. ID06-379 was observed after 5 days of incubation in optimization medium and Vogel's minimal medium as control. From the results, it was found that the extracellular β-mannanase production by *Nonomuraea* sp. ID06-379 revealed its best production at 2 days of incubation (5.2 U/mg) in modified optimization medium. At longer incubation periods the activity gradually decreased and at 5 days of incubation the β-mannanase activity was 30% lower than the activity obtained at 2 days of incubation (3.0 U/mg). The decrease in the production of mannanase by *Nonomuraea* sp. ID06-379 after 3 days of incubation period might be due to the depletion of nutrients and accumulation of other by-products like proteases in the fermentation medium initiating autolysis of cells (Meenakshi *et al.,* 2010; Malik *et al.*, 2010).

Conclusion

Hydrolysis mannanase by *Nonomuraea* sp. ID06-379 showed as an β-mannanase when galactomannans, glucomannans and β-mannans were used as sole substrates. Simple sugars supported the growth of the bacterium, but did not induce the mannanase production. The mannanase production seems to be more when LBG is used as a sole source of carbon. Malt extractf extract served as very good combination of nitrogen source. From the results observed, it can be concluded that optimization of nutritional components for the production of β-mannanase from actinomycete *Nonomuraea* sp. ID06-379 was successfully done. Attempts to clone this mannanase gene will be carried out in order to understand over production of β-mannanase secreted by *Nonomuraea* sp. ID06-379 that later may be useful in industrial application such as in food processing, biobleaching and biofuel.

Acknowledgements

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